Propionate in Heme Biosynthesis in Soybean Nodules

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Summary. When soybean nodules are incubated with propionate-2-14C the heme moiety of leghemoglobin becomes labeled. The incorporation of propionate-2-14C into heme is linear with time and it appears that propionate is utilized without a lag period. The rate of incorporation of propionate-2-14C into heme is more rapid than the rate of incorporation of succinate-2-14C and citrate-1,5-14C, however, these rates of incorporation may be influenced by different sizes of endogenous pools of organic acids.

Additional radioactive tracer experiments demonstrate that the supply of heme precursors from propionate is competitive with the supply of heme precursors from the citric acid cycle. When the concentration of propionate was high in the incubation mixture, the rate of succinate-2-14C incorporation into heme was decreased. Furthermore, when a large amount of succinate or acetate is added to the incubation mixture containing whole nodules, the rate of incorporation of propionate-2-14C into heme is reduced. The data support the hypothesis that propionate utilization makes possible a mechanism for the formation of succinyl-CoA in addition to that provided by the citric acid cycle.

The fact that propionate is readily utilized by bacteroids suggested that this compound may be a normal metabolite in nodules. No detectable pool of propionate was found, however, in either soybean nodules or in isolated bacteroids suggesting that propionate, if present, is utilized as rapidly as it is formed. Experiments in which cell-free extracts of nodule bacteroids were used demonstrated the conversion of lactate to propionate. The cofactor requirements for these enzymic reactions are adenosine 5-triphosphate, Mg++ and reduced nicotinamide adenine dinucleotide.

Cobalt is essential for *Rhizobium* species and symbiotically grown legumes (2, 16, 17, 18) but has not been shown to be essential for either leguminous or non-leguminous plants per se. The specific role or roles of cobalt in the metabolism of either symbiotic or free-living nitrogen-fixing organisms has not been adequately determined. De Hertogh et al. (10) have shown that propionate is activated in the presence of ATP and CoA and that the product, propionyl-CoA, is carboxylated yielding methylmalonyl-CoA and finally converted to succinyl-CoA via the methylmalonyl-CoA mutase reaction. Cobalt deficiency in *Rhizobium* cells is known to result in a decreased synthesis of B12 coenzyme which impairs the activity of the methylmalonyl-CoA mutase reaction (10). This lesion, apparently is responsible for the failure of cobalt deficient *Rhizobium* cells to effectively metabolize propionate.

There is evidence suggesting that vitamin B12 is involved in the biosynthesis of hemoglobin in animals (4, 7, 11), however, knowledge of the precise sites where the vitamin functions is incomplete. An involvement in some way of vitamin B12 in the biosynthesis of leghemoglobin was suggested by the demonstration (2) of a significant reduction in the content of nodule leghemoglobin when soybeans were cultured in media lacking adequate cobalt. Further, it has been shown that (15) the rates of increase in concentrations of B12 coenzyme and leghemoglobin are nearly parallel during the development of soybean nodules. Wilson and Reisenauer (25) have shown that the nodules of symbiotically-grown alfalfa contain only traces of leghemoglobin when 0.001 µg of cobalt per liter was supplied in the nutrient medium. When 0.010 µg of cobalt per liter was provided to alfalfa plants, the leghemoglobin content of the nodules increased strikingly.

By the use of specifically labeled glycine-14C and acetate-14C, Richmond and Salomon (21) have demonstrated that the general sequence of reactions

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leading to heme formation in soybean nodules is similar to that established for heme synthesis in animals. The pathway of acetate utilization in porphyrin synthesis is through the citric acid cycle which leads to succinyl-CoA formation. Succinyl-CoA is then condensed with glycine to form delta-aminolevulinate, the direct precursor of porphyrins.

In soybean nodules the activation of propionate and conversion to succinyl-CoA via methylmalonyl-CoA makes possible a second mechanism for the formation of succinyl-CoA (9, 10). The purpose of this investigation is to determine whether propionate may serve as a significant precursor for heme biosynthesis as well as for participation in the citric acid cycle. Since nothing is known about propionate biosynthesis in legume nodules, it was desired to obtain information on possible pathways of propionate formation.

**Material and Methods**

*Source of Chemicals.* Recrystallized hemin, GSH and the dipotassium salt of ATP were obtained from Nutritional Biochemicals Corporation. Sodium propionate-2-14C (5.15 mc/mmole), citrate-1,5-14C (5.32 mc/mmole), alpha-ketoglutarate-5-14C (6.94 mc/mmole), succinate-2-14C (9.1 mc/mmole) and alpha-aminolevulinate-4-14C (11.7 mc/mmole) were purchased from New England Nuclear Corporation and sodium lactate-1-13C (5.47 mc/mmole) and lactate-2-13C (11.7 mc/mmole) were obtained from the Volk Radiochemical Company. Coenzyme A was purchased from the Sigma Chemical Company and other chemicals and solvents used were reagent grade from commercial sources.

*Biological Materials.* The biological materials utilized in these experiments were soybeans (Glycine max Merr. variety Chippewa) and cowpeas (Vigna sinensis L. variety Iron clay mixed). The plants were grown in a greenhouse using the cultural methods described by Ahmed and Evans (12). Salts used in the nutrient solution were not purified. The nodules were harvested when plants were 38 to 48 days old. Precise ages of nodules are indicated in the legends of figures and tables.

*Separation and Identification of Organic Acids.* The ether extraction method of Swim and Utter (23) was utilized for the column chromatographic separation and identification of non-volatile organic acids in nodules and extracts. The identities of succinate, fumarate and malate that were isolated from a Celite column were confirmed by co-chromatography of these acids with known standards on thin-layer plates of silica gel G (24). For the separation of acids in nodule extracts by thin-layer chromatography, the method of Swim and Utter (23) was modified by passing the water extract of nodules, after evaporation of the ether, through a Dowex 50W-X8 cation exchange column to remove amino acids. The eluate from the column was evaporated to dryness and taken up in 0.5 ml of 95% ethanol. A 100 µl aliquot was applied to glass plates (24).

For the determination of the volatile fatty acids, fresh nodules were macerated in a mortar with an equal volume of water. Bacteroids from nodules were mixed with 10 ml of water and broken after freezing by use of an Eaton press. The homogenates of nodules or bacteroids were acidified to pH 2 with H2SO4 and subjected to steam distillation. The distillate was neutralized to pH 7 with 0.2 x NaOH and then evaporated to near dryness in a rotary evaporator. The residue from each sample was dissolved in 1.5 ml of 0.2 x H2SO4 and applied to a Celite column (23) for isolation of the acids. Butyric and propionic acids were eluted with 100% chloroform-, acetate with 95% chloroform and 5% n-hexyl alcohol, and formate with 90% chloroform and 10% n-hexyl alcohol. The identity of propionate was further established by use of propionate-2-14C. One µC of this compound was added to a standard mixture of volatile acids and it was shown that the peak of radioactivity corresponded with the propionate peak as determined by titration with NaOH.

*Isolation and Purification of Heme.* The method used for the isolation and purification of heme from legume nodules was developed from the methods described by Richmond, Altman and Salomon (20), Chu and Chu (5), Kiese and Kurz (14) and Hubler and Vishniac (13). Nodules were washed thoroughly and then macerated in a mortar with an equal volume of water. The residue was removed by centrifugation and the supernatant fraction was adjusted to pH 3 with glacial acetic acid. Sufficient acetone was added to the supernatant solution to make it 80% acetone. The precipitate was removed by centrifugation and 20 ml of chloroform were added to the aqueous acetone solution. After 2 hours at room temperature, the heme was extracted thoroughly with 20 ml aliquots of chloroform. After combined chloroform extracts were washed 4 times with 100 ml aliquots of distilled water, the chloroform phase was evaporated to dryness and the residue taken up in 0.5 ml pyridine. Chromatography of a 100 µl sample of this material revealed a fluorescent spot under ultra-violet light that is typical of porphyrins. In order to remove porphyrins and other contaminants from the heme sample in pyridine, sufficient water to make a 1% pyridine solution was added and the solution chromatographed on a silicone-impregnated cellulose column (13). The column was washed several times with water and the heme was eluted with pyridine-propanol-water (1:3:12.5) v/v. The solvent subsequently was removed by evaporation and the heme was taken up in 1.0 ml pyridine and used for determination of radioactivity, for measurement of heme content and for further chromatography. The specific activity of heme isolated by this pro-
procedure remained essentially constant in 3 successive chromatographic separations (table I). Further proof that the heme samples were relatively pure was obtained by chromatography of a representative sample of purified radioactive heme on paper (6) and scanning the paper with a strip counter. In this experiment radioactivity was limited to 1 spot corresponding to that of authentic heme. The spectrum of the pyridine hemochromogen from isolated samples was essentially identical to that of recrystallized hemin. Further identification was accomplished by the determination of the Rf values of heme samples in lutidine and in water-propanol-pyridine (5.5:0.1:0.4 v/v/v (6). The concentration of heme was determined by measurement of the optical density of reduced pyridine hemochromogen at 555 m" (12).

Measurement of Radioactivity. For the measurement of radioactivity in the heme moiety of leghemoglobin an aliquot containing 0.04 to 0.08 µmoles of purified heme was placed in a glass planchet and evaporated to dryness with a warm jet of air. The samples were counted at infinite thinness using a Nuclear Chicago model 181 B gas flow detector. The radioactivity was proportional to the quantity of heme added to each planchet in the range of 0.0 to 0.1 µmole of heme. The efficiency of the counting procedure was 12 to 15% and sufficient counts were recorded to obtain a standard deviation of 1.4% or less. In a typical experiment each 4 g sample of nodules contained 1.0 to 1.2 µmoles of heme. The amount of heme isolated from each sample ranged from 0.42 to 0.58 µmoles and the incorporation of propionate-2-14C and succinate-2-14C into heme was approximately 1% of that added to the reaction mixtures.

For the measurement of radioactivity in propionic and acetic acids the peak effluent fraction from a Celite column was neutralized and an aliquot containing 0.2 to 0.6 micro equivalent was added to a glass planchet, evaporated to dryness and counted at infinite thinness. In a single experiment 14CO2 was collected, converted into BaCO3, plated and assayed for radioactivity at saturation thickness (19).

Standard Incubation Procedure. Nodules were harvested, washed in ice water and samples of 4 g fresh weight were weighed for use in isotope incorporation experiments. Each sample unless otherwise indicated in legends of tables and figures was placed in 6.0 ml of 0.1 M potassium phosphate buffer (pH 5.6). This pH was shown to be optimum for incorporation of propionate-2-14C in whole nodules. To the 6.0 ml of buffer solution were added 50 µmoles glutamate, 12 µmoles glycine, 5 µc of 14C-labeled metabolite and 12 µmoles of the appropriate non-radioactive metabolite to minimize the effect of endogenous pools of metabolites. To aid in the introduction of the reactants into the nodule tissues, each sample of nodules immersed in the incubation medium was evacuated at 70 mm Hg for 3 minutes. The use of boiled nodule extracts in place of fresh extracts in reaction mixtures resulted in no measurable incorporation of radioactivity into the heme of leghemoglobin and thus control reactions using boiled extracts or nodules are not presented for each individual experiment.

Preparation of Cell-Free Extracts. Cell-free extracts were prepared by freezing the nodules in an equal volume of 0.1 M potassium phosphate buffer (pH 7.0) in solid CO2 and breaking the cells in an Eaton press at a pressure of 8000 pounds per square inch. The cell debris was removed by centrifugation and the extract dialyzed under argon for 5 to 6 hours in 2 liters of 0.1 M potassium phosphate buffer (pH 7.0).

For the preparation of cell-free extracts of bacteroids, nodules were macerated in 2 volumes of 0.1 M phosphate buffer at pH 7.0, squeezed through 4 layers of cheesecloth to remove the solid debris and the bacteroids collected by centrifugation. The bacteroids were washed repeatedly with 40 ml volumes of 0.1 M phosphate buffer at pH 7.0 until all traces of hemoglobin were removed. A slurry of the bacteroids (1 wt per 1 vol of 0.1 M phosphate buffer at pH 7.0) was placed in an Eaton pressure cell, frozen in solid CO2 and broken as described for nodules. After centrifugation, the cell-free extract was dialyzed as described for nodules and stored under argon at -70°. The protein determination was made by the Biuret method (8).

Results

Organic Acids in Nodules. Since propionate is readily oxidized by the bacteroids from nodules and

| Table I. Purity of Radioactive Heme Isolated from Cowpea Nodules |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Times chromographed (No)  | Heme isolated (muoles) | Specific activity of heme (cpm/umole) | Standard deviation |
| 1 | 0.54 | 27,900 | ± 279 |
| 2 | 0.30 | 27,800 | ± 278 |
| 3 | 0.18 | 27,300 | ± 273 |
the enzymes for propionate utilization are present in nodule bacteroids (10) propionate might be expected to be present as a normal metabolite in soybean nodules. Investigations were carried out, therefore, to determine the content of propionate and other organic acids in nodules. The data presented in table II show that soybean nodules contain a relatively high concentration of acetic acid, measurable quantities of butyric and formic acids but no detectable quantity of propionic acid. Even though radioactive propionate added to nodule homogenates could be recovered satisfactorily, no evidence was obtained by either column chromatography or gas chromatography that propionate was present in soybean nodules.

Data in table II also indicate that nodules from soybean plants contain several citric acid cycle acids. Malic acid is present in the highest concentration and appreciable amounts of α-ketoglutaric, succinic, fumaric and isocitric acids were found. In this analysis no citric acid was detected, however, in other experiments a trace of this acid was found. The identity of the various citric acid cycle acids isolated by column chromatography was confirmed by subjecting a sample of each acid to thin-layer chromatography using the procedure of Ting and Dugger (24). Thin-layer chromatography of nodule extracts consistently revealed 2 additional acids with Rf values of 0.09 and 0.02 but these compounds were not identified.

In another experiment, 4 g of nodules were incubated with 5 μc propionate-2-14C for 1 hour following the standard incubation procedure. Citric acid cycle acids were separated by thin-layer chromatography and plates were scanned for radioactivity. The results showed that radioactivity was incorporated into fumarate, succinate, α-ketoglutarate and malate. These data confirm the observations of De Hertogh et al. (10) and provide evidence that propionate may serve as an alternate carbon source for maintenance of the citric acid cycle.

**Incorporation of 14C-labeled Metabolites into Heme.** From the data presented graphically in figure 1 it is apparent that the heme moiety of leg-hemoglobin becomes radioactive when nodules are incubated with propionate-2-14C. The rate of incorporation of 14C into heme from propionate-2-14C is linear with time and appears to occur without a lag period. Similar results were obtained in other experiments using nodules from cowpea plants. Sliced, homogenized or whole soybean nodules, or cell-free extracts of soybean nodules, effectively incorporated labeled propionate into heme but subjection of nodules or extracts to 100° for 3 minutes prevented the incorporation.

Since propionate functioned as a heme precursor, additional experiments were needed to determine the rate of propionate incorporation into heme in relation to the rates of incorporation of other metabolites. In a series of replicate experiments the relative rates of incorporation of radioactive organic acids have been reproducible and have shown that propionate is incorporated into heme at a faster rate than either citrate or succinate (fig 1). In other experiments the rate of incorporation of 14C from α-ketoglutarate-5-14C was slightly greater than that of propionate-2-14C and the initial rate of incorporation of fumarate-2,3-14C was comparable to that of succinate-2,3-14C. In experiments comparable to that described in figure 1, δ-aminolevulinate-4-14C was very rapidly incorporated into heme. The specific activity of the isolated heme after 1, 3, 5 and 7 hours of incubation was 1240, 6700, 13,900 and 20,600 cpm respectively. This rate was linear with time.

In an effort to avoid complications in interpretation of rates of incorporation of metabolites into heme resulting from different pool sizes of endogenous metabolites, experiments were carried out using cell-free extracts of soybean nodules. From the data in table III it appears that α-ketoglutarate-5-14C is converted into heme approximately 4 to 5 times faster than either propionate-2-14C or propionate-1-14C. Decreasing the enzyme extract by one-half (table III Experiment 1) decreased the rate of incorporation of propionate-2-14C into heme by 40%. The addition of 12 μmoles unlabeled carrier significantly reduced the rate of incorporation of propionate-2-14C or succinate-2,3-14C into heme. Dialysis of the extract (table III Experiment 2) resulted in a 2-fold increase in the rate of incorporation of α-ketoglutarate-5-14C and lactate-1-14C into heme and increased the rate of incorporation of δ-aminolevulinate-4-14C by approximately 23%. On the other hand, dialysis failed to significantly influence the rate of incorporation of propionate-1-14C. The low rate of incorporation of propionate might have been caused by lability.

### Table II. Organic Acid Content of Soybean Nodules

Volatile fatty acids in a 20 g sample of soybean nodules (42 days old) and citric acid cycle acids in a 10 g sample of soybean nodules (40 days old) were determined by the procedures described in Materials and Methods.

<table>
<thead>
<tr>
<th>Acid</th>
<th>Concentrations (micro equivalents per g fr wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Volatile acids</strong></td>
<td></td>
</tr>
<tr>
<td>Formic</td>
<td>0.67</td>
</tr>
<tr>
<td>Acetic</td>
<td>11.89</td>
</tr>
<tr>
<td>Propionic</td>
<td>0.00</td>
</tr>
<tr>
<td>Butyric</td>
<td>0.82</td>
</tr>
<tr>
<td><strong>Citric acid cycle acids</strong></td>
<td></td>
</tr>
<tr>
<td>Fumaric</td>
<td>2.32</td>
</tr>
<tr>
<td>Succinic</td>
<td>3.77</td>
</tr>
<tr>
<td>α-Ketoglutaric</td>
<td>6.10</td>
</tr>
<tr>
<td>Malic</td>
<td>20.19</td>
</tr>
<tr>
<td>Citric</td>
<td>0.00</td>
</tr>
<tr>
<td>Isocitric</td>
<td>2.90</td>
</tr>
</tbody>
</table>
of enzymes involved in propionate utilization. From the data in Table III, it appears that lactate can also function as a heme precursor.

**Competing Pathways for the Formation of Heme**

Precursors. Since a supply of succinyl CoA for heme synthesis could arise from either intermediates of the citric acid cycle or from the utilization of propionate, it would be logical to expect that the addition of non-radioactive propionate would result in an increased supply of non-radioactive succinyl-CoA and thus dilute the incorporation into heme of radioactivity from labeled intermediates of the citric acid cycle. Table IV shows that the addition of 12 μmoles of non-radioactive propionate to the incubation mixture resulted in a substantial reduction in the rate of incorporation of succinate-2-14C into heme. In 4 different experiments the trends in the results were similar. Furthermore in reciprocal experiments where 12 μmoles of non-radioactive succinate were added to an incubation mixture the rate of incorporation of 14C from propionate-2-14C was reduced to an extent of 40 to 60%.

The addition of non-radioactive acetate to either whole or macerated nodules resulted in a decreased rate of incorporation of propionate-2-14C into heme. The effect of adding 12 μmoles of non-radioactive acetate to each reaction mixture on the rate of incorporation into heme of propionate-2-14C and succinate-2-14C is illustrated in Figure 2. The addition of unlabeled acetate reduced by approximately 33% the rate of incorporation of propionate-2-14C.

**Table III. Incorporation of 14C-Labeled Precursors into Heme in Cell-free Extracts from Soybean Nodules**

Experiment 1. Cell-free extract was prepared from 40 day-old soybean nodules and dialyzed for 6 hours in 0.1 M phosphate buffer (pH 7.0). The reaction mixture in a final volume of 6 ml 0.1 M phosphate buffer at pH 5.6 contained 2 μc 14C-labeled metabolite; 10 μmoles of ATP and MgCl₂; and 0.2 μmole of CoA and NADH. Twelve μmoles carrier were added as indicated. The incubation mixture contained 20 mg protein/ml unless indicated. Tubes were incubated 90 min at 35°C. Radioactivity incorporated into heme using a boiled extract (2 cpm/0.07 μmole heme) was subtracted from each determination.

Experiment 2. Same as in experiment 1 except that the cell-free extract was prepared from 28 day-old nodules. Each reaction mixture contained 0.26 μmole heme from extract and was incubated for 50 min at 38°C at pH 7.0.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Extract</th>
<th>Unlabeled carrier added (μmoles)</th>
<th>Specific activity of heme (cpm/μmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propionate-2-14C</td>
<td>dialyzed</td>
<td>0</td>
<td>458</td>
</tr>
<tr>
<td>Propionate-2-14C</td>
<td>dialyzed</td>
<td>12</td>
<td>211</td>
</tr>
<tr>
<td>Propionate-2-14C</td>
<td>dialyzed</td>
<td>0</td>
<td>270</td>
</tr>
<tr>
<td>Succinate-2,3-14C</td>
<td>dialyzed</td>
<td>0</td>
<td>1316</td>
</tr>
<tr>
<td>Succinate-2,3-14C</td>
<td>dialyzed</td>
<td>12</td>
<td>267</td>
</tr>
<tr>
<td>α-ketoglutaric-5-14C</td>
<td>dialyzed</td>
<td>0</td>
<td>2285</td>
</tr>
<tr>
<td>Lactate-2-14C</td>
<td>dialyzed</td>
<td>0</td>
<td>540</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment 2</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Propionate-1-14C</td>
<td>crude</td>
<td>12</td>
<td>92</td>
</tr>
<tr>
<td>Propionate-1-14C</td>
<td>dialyzed</td>
<td>12</td>
<td>89</td>
</tr>
<tr>
<td>α-ketoglutaric-5-14C</td>
<td>crude</td>
<td>12</td>
<td>189</td>
</tr>
<tr>
<td>α-ketoglutaric-5-14C</td>
<td>dialyzed</td>
<td>12</td>
<td>380</td>
</tr>
<tr>
<td>δ-aminolevulinic acid-4-14C</td>
<td>crude</td>
<td>12</td>
<td>1720</td>
</tr>
<tr>
<td>δ-aminolevulinic acid-4-14C</td>
<td>dialyzed</td>
<td>12</td>
<td>2132</td>
</tr>
<tr>
<td>Lactate-1-14C</td>
<td>crude</td>
<td>12</td>
<td>290</td>
</tr>
<tr>
<td>Lactate-1-14C</td>
<td>dialyzed</td>
<td>12</td>
<td>658</td>
</tr>
<tr>
<td>Lactate-1-14C + 20 μmoles unlabeled propionate</td>
<td>dialyzed</td>
<td>12</td>
<td>452</td>
</tr>
</tbody>
</table>

1 Reaction mixture contained 10 mg protein/ml.
into heme and increased by about 50\% the rate of incorporation into heme of radioactive succinate.

**Pathways of Propionate Formation.** Determination of the rate of conversion of \(^{14}C\)-labeled lactate into heme in whole nodules provided an indication that propionate may be an intermediate in lactate utilization (table V). Both lactate-\(^{2-14}C\) and lactate-\(^{1-14}C\) were converted into the heme moiety of leghemoglobin at nearly equal rates. The addition of unlabeled propionate to the reaction mixture (table V) resulted in a reduction in the rate of \(^{14}C\) incorporation into heme from both lactate-\(^{1-14}C\) (59\%) and lactate-\(^{2-14}C\) (46\%). When the remaining propionate was isolated and assayed, it was apparent that \(^{14}C\) from radioactive lactate was incorporated into propionate. Since the duration of these experiments was 5 and 8 hours, the label could have been converted into propionate via an indirect route. The data presented in table II using a cell-free extract instead of whole nodules shows that the addition of 20 \(\mu\)moles of propionate reduced the rate of incorporation of lactate-\(^{1-14}C\) into heme by approximately 30\%. This further supports the hypothesis that propionate is an intermediate in the conversion of lactate into heme.

By the use of cell-free extracts from nodule bacteroids, the enzymatic conversion of lactate into propionate was studied. Data in table VI show that the conversion of lactate to propionate in reaction mixtures containing nodule extracts required ATP, CoA, NADH and Mg\(^{++}\) as cofactors. The time course for the conversion of lactate-\(^{1-14}C\) into propionate was found to be linear for a period of 30 minutes (fig 3). The rate of conversion of lactate-\(^{2-14}C\) into propionate was approximately equal to the rate of conversion of lactate-\(^{1-14}C\) into propionate. These results are in harmony with those presented in table V. In another experiment the addition of 10 \(\mu\)moles acrylate to an incubation mixture identical with the complete reaction mixture described in table VI reduced by 54\% the rate of formation of radioactive propionate from lactate-\(^{1-14}C\). These results are consistent with the possibility that acrylate may be an intermediate in the pathway.

The rate of conversion of radioactive lactate into acetate and CO\(_2\) also was followed in an experiment using a cell-free extract and the complete reaction mixture in table V. The loss of radioactivity from lactate-\(^{1-14}C\) as \(^{14}CO_2\) was appreciable (330 cpm) after a 20 minute incubation period at \(38^\circ\) while very little CO\(_2\) became labeled from lactate-\(^{2-14}C\) (17 cpm). During this time, the \(^{14}C\)

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**Table IV. Effect of Adding Propionate on the Rate of Incorporation of Succinate-\(^{2-14}C\) into Heme in Soybean Nodules**

Intact 39 day-old soybean nodules were utilized following the conditions of the standard incubation procedure. Five \(\mu\)c of succinate-\(^{2-14}C\) were added to all reaction mixtures and non-radioactive propionate was added as indicated. Incubation was carried out on a shaker at 26 to 28\°.

<table>
<thead>
<tr>
<th>Hours incubated</th>
<th>Propionate added ((\mu)moles)</th>
<th>Sp activity of heme (cpm/(\mu)mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0</td>
<td>338</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>182</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>440</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>313</td>
</tr>
</tbody>
</table>

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**Table V. Effect of Adding Propionate on the Rate of Incorporation of Lactate-\(^{14}C\) into Heme in Soybean Nodules**

Nodules were incubated following the procedure outlined in the Materials and Methods with the exception that propionate was added as indicated. To each reaction mixture was added 12 \(\mu\)moles of lactate containing 5 \(\mu\)c of \(^{14}C\).

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Age of nodules (days)</th>
<th>Hrs incubated</th>
<th>Propionate added ((\mu)moles)</th>
<th>Sp activity of heme (cpm/(\mu)mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate-(^{2-14}C)</td>
<td>44</td>
<td>5</td>
<td>0</td>
<td>841</td>
</tr>
<tr>
<td>Lactate-(^{1-14}C)</td>
<td>44</td>
<td>5</td>
<td>12</td>
<td>457</td>
</tr>
<tr>
<td>Lactate-(^{1-14}C)</td>
<td>39</td>
<td>8</td>
<td>0</td>
<td>1289</td>
</tr>
<tr>
<td>Lactate-(^{1-14}C)</td>
<td>39</td>
<td>8</td>
<td>12</td>
<td>529</td>
</tr>
</tbody>
</table>
accumulation in acetate from lactate-2-14C was much greater (1565 cpm per μmole acetate) than that from lactate-1-14C (227 cpm per μmole acetate). This difference in the rate of label accumulation was not apparent when lactate-1-14C and lactate-2-14C were converted into propionate.

![Graph](https://via.placeholder.com/150)

**Fig. 3.** The rate of conversion of lactate-1-14C into propionate by a cell-free extract of bacteroids from soybean nodules. The extract was prepared from bacteroids from 28 day-old nodules. The final volume of 1.5 ml contained 96 μmoles phosphate buffer (pH 7.0); 0.1 ml extract (14 mg protein); 2 μC lactate-1-14C in 20 μmoles non-radioactive lactate; 10 μmoles each of propionate, ATP, and MgCl2; 0.2 μmole CoA and NADH. No incorporation occurred when boiled extract was used in place of above extract.

**Table VI. Cofactor Requirements for Lactate Utilization by a Cell-free Extract from Nodule Bacteroids**

The complete reaction mixture in a final volume of 1.57 ml of 0.064 M phosphate buffer pH 7.0 contained 2 μC lactate-1-14C (5.47 μC/μmole) and the following in μmoles: unlabelled lactate, 20; unlabelled propionate, 10; ATP, 10; MgCl2, 10; NADH, 0.2; CoA, 0.2 and 0.1 ml enzyme extract containing 14 mg protein. Each reaction mixture was incubated at 36°C for 20 min. Complete reaction mixture with enzyme extract omitted served as a negative control.

<table>
<thead>
<tr>
<th>System</th>
<th>Enzyme activity (total cpm incorporated into 10 μmoles propionate in 20 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-CoA</td>
<td>210</td>
</tr>
<tr>
<td>-NADH</td>
<td>330</td>
</tr>
<tr>
<td>-Mg2+</td>
<td>320</td>
</tr>
<tr>
<td>-ATP</td>
<td>400</td>
</tr>
</tbody>
</table>

**Discussion**

Experiments have been conducted to test the hypothesis that propionate serves as an alternate carbon source for the supply of succinyl-CoA that may be utilized either for heme biosynthesis or for the maintenance of the citric acid cycle. The report by De Hertog et al. (10) that propionate-1-14C is converted into intermediates of the citric acid cycle has been confirmed.

Radioactive tracer experiments have demonstrated that propionate can be incorporated into the heme component of leghemoglobin in intact nodules or nodule extracts (table III, fig 1). It appears that propionate is utilized without a lag period and that the rate of 14C incorporation into heme is linear with time. The fact that propionate-2-14C not only is incorporated into intermediates of the citric acid cycle, but also serves as a precursor of the heme moiety of leghemoglobin, is consistent with the hypothesis that this compound makes possible a second mechanism for the formation of succinyl-CoA. Admittedly the rates of incorporation of 14C labeled propionate into heme are relatively low but, they are equal to or higher than the rates of incorporation of several citric acid cycle intermediates. It has been established (10) that cobalt deficiency in Rhizobium meliloti results in a limited supply of B12 coenzyme and that this lesion blocks the synthesis of succinyl CoA from propionate. The possibility is presented therefore that reduced leghemoglobin contents of cobalt deficient soybean nodules is caused by a failure to efficiently utilize propionate. The significance of relative rates of incorporation of metabolites into heme is difficult to determine because of the different sizes of endogenous organic acid pools that are present and the lack of information regarding equilibration of internal pools with external additions. No data were obtained proving net synthesis of heme during the incubation periods in either whole nodules or in cell-free extracts of nodules and the possibility that 14C accumulation in the heme moiety of leghemoglobin occurred from exchange reactions mediated by the reversible enzymes of the system has not been ruled out. It was established that incorporation of radioactive propionate and other metabolites into heme proceeded enzymatically since no measurable 14C was incorporated when nodules or nodule extracts were boiled.

Since propionate did not occur in nodules in detectable amounts (table 1) it must be concluded that the acid, if present, is utilized as rapidly as it is formed. A search was initiated, therefore, to identify possible precursors. It has been reported (22) that the interior of nodules is nearly anaerobic and that lactate may be fermented by nodule bacteroids (5). This information suggested that the pathway where lactate is converted to propionate via acrylate (3) may be operative. Results pre-
sented in tables IV and V are consistent with the postulation that lactate may serve as a precursor of propionate. Experiments in which cell-free extracts of lactic acid bacteria were utilized provided convincing evidence for the enzymatic conversion of lactate to propionate (table V). The addition of the possible intermediate, acrylate, to the reaction mixture reduced by approximately 50% the rate of conversion of lactate-1-14C into propionate, but the possibility that acrylate was inhibitory has not been ruled out.

The rate of conversion of lactate-1-14C into propionate and into the heme moiety of leghemoglobin is approximately equal to the rate of conversion of lactate-2-14C into these compounds. This would not be expected if the major pathway involved a conversion of lactate to pyruvate and then to propionate after decarboxylation. From the results presented in table VI it seems highly probable that a major portion of the added lactate is converted to propionate via a route involving lactyl CoA and acrylyl CoA as intermediates (3). The conversion of some lactate to pyruvate is supported by the fact that 14C from lactate-1-14C and lactate-2-14C was enzymatically converted to acetic acid and CO2 under anaerobic conditions. Much of the label from lactate-1-14C, however, was lost as 14CO2 thus causing much greater accumulation of 14C in acetate from lactate-2-14C than from lactate-1-14C.

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Literature Cited